Comparison of the Genetic Recombination Rates of Human Immunodeficiency Virus Type 1 in Macrophages and T Cells†

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Human immunodeficiency virus type 1 (HIV-1) exhibits a high level of genetic variation generated by frequent mutation and genetic recombination during reverse transcription. We have measured HIV-1 recombination rates in T cells in one round of virus replication. It was recently proposed that HIV-1 recombines far more frequently in macrophages than in T cells. In an attempt to delineate the mechanisms that elevate recombination, we measured HIV-1 recombination rates in macrophages at three different marker distances. Surprisingly, the recombination rates were comparable in macrophages and in T cells. In addition, we observed similar recombination rates in two monocytic cell lines regardless of the differentiation status. These results indicate that HIV-1 undergoes similar numbers of recombination events when infecting macrophages and T cells.

Human immunodeficiency virus type 1 (HIV-1), like most other retroviruses, packages two copies of viral RNA into one virion (8, 17). Reverse transcriptase can use portions of the genomes from each RNA as templates to generate a recombinant viral DNA copy during reverse transcription (6, 11, 12). Although recombination can occur in all virions, a genetically different progeny virus can be generated only from virions containing two RNAs encoding different genetic information (heterozygous virions) (11). Heterozygous virions are generated only from cells infected with more than one retrovirus (double infection). Our recent studies demonstrated that double infection occurs frequently in HIV-1 infection, which provides the basis for heterozygous virion formation and subsequent generation of recombinant viruses (7). The inherent ability of HIV-1 to recombine and generate variation in viral populations affects at least two aspects of viral pathogenesis. The variability of the viral populations is a challenge for the immune system of the infected individual and for the antiviral treatments to control virus replication, because escape mutants and drug-resistant variants can easily emerge from the viral population (5, 19).

HIV-1 recombination occurs frequently and throughout the viral genome, although some regions may experience more recombination events than others (4, 9, 13, 14, 18, 20, 21, 23, 25, 27). HIV-1 also recombines at a much higher frequency than other retroviruses (1, 11, 22, 25); in one round of replication with two markers 1 kb apart, the recombination rates of spleen necrosis virus and murine leukemia virus are 4.0 and 4.7%, respectively (1, 11); in contrast, the recombination rate of HIV-1 is 42.4% (25).

To study HIV-1 recombination in its natural target cells, we developed a system that used flow cytometry to detect virus infection and recombination and measured HIV-1 recombination rates in activated human primary CD4+ T cells and a human T-cell line at three different marker distances (24). A recent report concludes that HIV-1 recombines three- to fivefold more frequently in macrophages than in T cells or fibroblasts (20). Because macrophages are one of the natural target cells for HIV-1 (2, 26), understanding the molecular mechanisms of HIV-1 infection of macrophages, including HIV-1 recombination in macrophages, is important for the elucidation of HIV-1 infection and pathogenesis. In this report, we sought to delineate the molecular mechanisms that elevate the HIV-1 recombination rate in macrophages. In contrast to the previous report, we observed that HIV-1 recombines at similar rates when infecting macrophages or T cells.

System used to measure HIV-1 recombination rates. As previously described (24), a pair of HIV-1 vectors was used in each recombination assay. Each vector encodes two marker genes: the first gene is either a mouse heat-stable antigen gene (*HSA*) (16) or the mouse CD90.2 gene (also known as *Thy-1*) (10), and the second gene is a modified green fluorescent protein gene (*GFP*) (3) with an inactivating mutation. The expressions of HSA and Thy-1 are used to monitor virus infection. A functional *GFP* gene can be reconstituted by recombination; thus, GFP expression is used to monitor recombination events. By placing the inactivating mutations at different positions in *GFP*, we were able to measure recombination rates at genetic distances of 103, 288, and 588 bp during one round of HIV-1 replication (24).

Measuring HIV-1 recombination rates in primary human macrophages. Human monocytes were isolated on the day of blood collection from healthy donors by elutriation at the National Institutes of Health Clinical Center. More than 95% of the elutriated cells expressed monocytic cell surface markers CD13 and CD14 (4, 15) on the day of isolation, which was determined by staining the cells with anti-CD13 and -CD14

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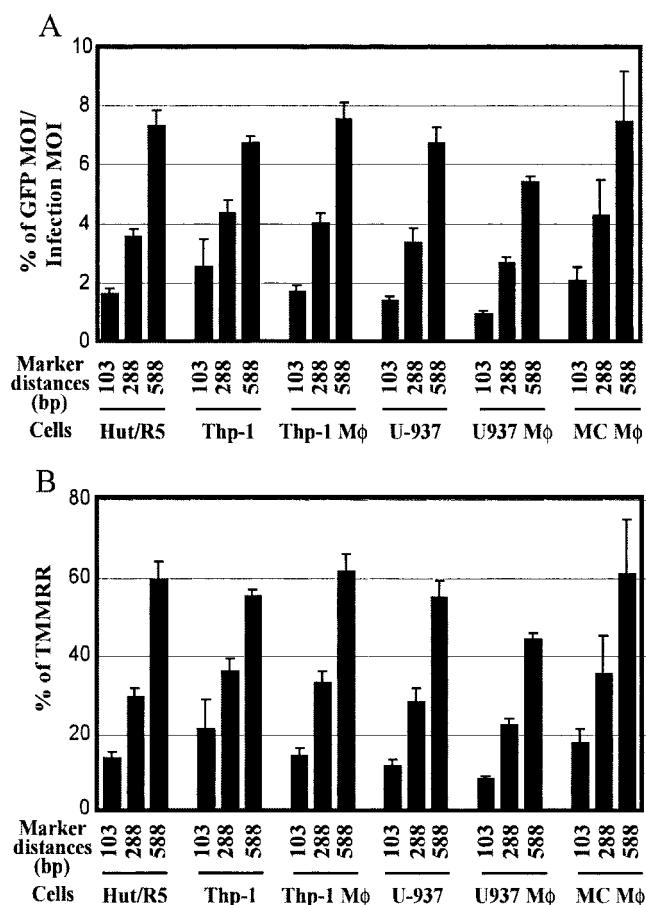† Supplemental material for this article may be found at http: //jvi.asm.org/.

A

B

FIG. 1. Comparison of HIV-1 recombination rates in different cell types. Recombination rates in different cell types are shown as percentages of GFP MOI/infection MOI (A) and percentages of theoretical maximum measurable recombination rates (B). Hut/R5, Hut/CCR5; Thp-1 Mϕ, Thp-1-derived macrophage; U937 Mϕ, U937-derived macrophage; MCMϕ, primary human monocyte-derived macrophage.

monoclonal antibodies (BD Biosciences) and flow cytometry analysis (data not shown). Primary macrophages were prepared from these monocytes by culturing the cells in RPMI 1640 medium containing 10% human AB serum for 6 days; recombinant human granulocyte-macrophage colony-stimulating factor was added at a final concentration of 500 U/ml in some experiments. Virus infection was performed in the presence of 10 μg/ml polybrene for 3 h; after the infection procedure, viruses were removed and fresh medium was added. Cells were detached from the culture plates at 6 days postinfection, stained with phycoerythrin-conjugated anti-HSA antibody (BD Biosciences) and allophycocyanin-conjugated anti-Thy1.2 (Thy-1) antibody (eBioscience), and analyzed by flow cytometry. Additionally, in some experiments, portions of the cells were stained with different combinations of anti-HSA, -Thy-1, -CD13, or -CD14 prior to analyses; our results demonstrated that almost all of the infected cells expressed monocytic cell marker(s) (data not shown). Data from five independent sets of experiments, each with cells from different donors, are summarized in Fig. 1 (also see Table S1 in the supplemental material).

As previously described, to calculate the recombination rate, the numbers of infected cells or GFP$^+$ cells were converted into multiplicities of infection (MOIs) (24). We estimated that the maximum GFP$^+$ MOI/infection MOI ratio would be 12.5%. This ratio was calculated as follows: from dually infected cells, with equal expression of the two parental proviruses and random RNA copackaging, 50% of the viruses produced would be heterozygous. If recombination between two markers occurred at the highest possible frequency, which would result in the random assortment of the two markers, then the heterozygous viruses would generate four evenly distributed *GFP* genotypes: *GFP* with a mutation from one parent, *GFP* with a mutation from the other parent, *GFP* with both mutations, and *GFP* with neither mutation. Of these four genotypes, only *GFP* with neither mutation would have the GFP$^+$ phenotype; thus, the theoretical maximum ratio of GFP$^+$ MOI/infection MOI would be 50%/4, or 12.5%. Previously, we also calculated the recombination rate as the percentage of the theoretical measurable maximum recombination rate (TMMRR) by dividing the observed GFP$^+$ MOI/infection MOI ratio by 12.5%.

In the experiments using macrophages as targets, the mean ratios of GFP$^+$ events in the infection events varied according to the distances between the two markers; they were 2.1, 4.4, and 7.6% with markers separated by 103, 288, and 588 bp, respectively (Fig. 1, MC Mϕ). These ratios also corresponded to 16.9, 34.8, and 60.7% of the TMMRR, respectively. Therefore, the recombination rates of HIV-1 in primary macrophages were similar to those observed in primary CD4$^+$ T cells and the Hut/CCR5 cell line (11.6, 30.7, and 55.9% of the TMMRR, respectively) (24).

Recombination rates in monocytic cell lines and macrophages derived from these cells. To determine whether macrophage differentiation affects the HIV-1 recombination rate as concluded by the other report (20), we examined the recombination rates in monocytic cell lines. We used the two monocytic cell lines described in the other study, U937 and Thp-1. These cells were obtained from the American Type Culture Collection and maintained as recommended by the provider. To induce macrophage differentiation, Thp-1 and U937 cells were cultured in the presence of phorbol 12-myristate 13-acetate at 20 ng/ml for 5 days, and their morphology was verified by microscopy.

Because phorbol 12-myristate 13-acetate downmodulates CD4 in U937 and Thp-1 cells (20), HIV-1 pseudotyped with vesicular stomatitis virus G protein (VSV G) was used to infect U937, Thp-1, and macrophages derived from these cells. Since our previous recombination experiments were performed using viruses pseudotyped with HIV-1 Env, to ensure that the recombination rates were not drastically altered for viruses pseudotyped with VSV G, we measured their recombination rates in Hut/CCR5 cells. We observed that 1.6, 3.6, and 7.4%, of the infection events exhibited the GFP$^+$ phenotype, which corresponded to 13.0, 28.9, and 59.4% of the TMMRR for markers separated by 103, 288, and 588 bp, respectively (Fig. 1, HutR5; also see Table S2 in the supplemental material). These rates are similar to those generated by viruses pseudotyped with CCR5-tropic HIV-1 Env in Hut/CCR5 cells (24). Additionally, we measured the recombination rates of VSV G-pseudotyped viruses in primary macrophages (Table S1 in the

supplemental material, experiment 5) and observed similar recombination rates. Therefore, the route of viral entry did not significantly affect recombination rates.

VSV G-pseudotyped HIV-1 stocks were used to infect U937 and Thp-1 cells and macrophages derived from these cells. Results from three independent sets of experiments are summarized in Fig. 1 (also see Tables S3 and S4 in the supplemental material). When U937 cells were used as targets, 1.4, 3.4, and 6.9% of the infection events resulted in the GFP$^+$ phenotype with markers separated by 103, 288, and 588 bp, respectively. When macrophages derived from U937 cells were used as target cells, 1.0, 2.7, and 5.5% of the infection events resulted in the GFP$^+$ phenotype with the same marker distances. These two sets of values are similar to one another, and they are similar to those measured in primary macrophages and T cells.

We also performed recombination experiments using Thp-1 cells and macrophages derived from Thp-1 cells as target cells. In Thp-1 cells, 2.6, 4.4, and 6.9% of the infection events yielded the GFP$^+$ phenotype; in Thp-1-derived macrophages, 1.7, 4.1, and 7.7% of the infection events yielded the GFP$^+$ phenotype with markers separated by 103, 288, and 588 bp, respectively (Fig. 1, Thp-1 and Thp-1 Mφ) (see Table S4 in the supplemental material). Therefore, we did not observe a marked increase in recombination frequency when U937 or Thp-1 cells were differentiated into macrophages.

HIV-1 recombination rates in T cells and macrophages. In this report, we measured HIV-1 recombination rates for markers separated by 103, 288, and 588 bp in different target cells including human primary macrophages, two monocytic cell lines, and macrophages derived from the two monocytic cell lines. In contrast to a previous report (20), we did not observe significantly higher recombination rates in primary human macrophages than in the Hut/CCR5 T cell line or primary T cells. We also did not observe differentiation-dependent enhancement of HIV-1 recombination in monocytic cells.

We sought to determine whether technical differences between our system and the other study could have resulted in the differences in our conclusions. A system based on flow cytometry was used to determine recombination frequency in the previous report, which described an increased recombination rate in macrophages (20). In that system, two parental viruses were used, one expressing yellow fluorescent protein (*YFP*) and one expressing cyan fluorescent protein (*CFP*); *YFP*, *CFP*, and *GFP* have similar nucleotide sequences. According to the report, *YFP* contains a T203Y mutation and *CFP* contains a Y67W mutation to confer the change of fluorescence wavelength. After recombination, a modified gene with neither mutation can be generated (termed *GFP**) that confers the GFP$^+$ phenotype via recombination (20). When Jurkat T cells were used as target cells, 8 to 9% of the infected cells were GFP$^+$; in contrast, 28.9%, 35%, and 37.3% of the infected cells were GFP$^+$ in primary macrophages and Thp-1- and U973-derived macrophages, respectively. From these results, it was concluded that HIV-1 recombines far more frequently in macrophages than in T cells. However, we found these results puzzling because we estimate that the theoretical maximum ratio of the GFP$^+$ phenotype to infection event should be 12.5% or 14.3%. The calculation is described as follows: with equal expression and random RNA copackaging, 50% of the virions

could be heterozygous. Since the phenotypes of the fluorescent protein genes are determined by the T203Y and Y67W mutations, if the mutations assort randomly, four genotypes would be generated at equal frequencies (12.5% each): the gene with the T203Y mutation (*YFP*), the gene with the Y67W mutation (*CFP*), the gene with neither mutation (*GFP**), and the gene with both mutations. It was not clear from the report whether the gene with both mutations could be detected by flow cytometry; if it could be detected either as YFP or CFP, then the GFP$^+$ phenotype would be generated at 12.5% of the infection event. In contrast, if the gene with both T203Y and Y67W mutations could not be detected, then 14.3% [12.5%/(100% − 12.5%)] of the progeny would have the GFP$^+$ phenotype. Given these estimations, it is rather perplexing that 28 to 37% of the infection yielded the GFP$^+$ phenotype in various macrophages, because these values were two- to threefold higher than the theoretical maximum observable rates.

In our assay system, we measured the HIV-1 recombination rates at three marker distances in each type of target cell. In all of these cells, as the marker distances increased, higher recombination rates were observed; furthermore, none of the rates exceeded the theoretical measurable maximum recombination rates, indicating the measured rates are within the dynamic range of the system. Therefore, results from our report indicate that HIV-1 recombines at comparable frequencies when infecting T cells and macrophages. Through these studies, we sought to understand the contribution of recombination to the variation of the HIV-1 viral population and ultimately help to control these pathogens.

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